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Cofactor Recycling Using a Thermostable NADH Oxidase

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1. Introduction

From the standpoint of enzymatic organic synthesis, NADH oxidase (NOX) will be a key enzyme that plays an essential role in the cofactor regeneration of NAD^+ dependent enzymatic reactions. For example, enzymatic enantioselective oxidations of racemic secondary alcohols (Geueke et al., 2003; Riebel et al., 2003; Hummel & Riebel, 1996) and amino acids (Hummel et al., 2003a) have been reported as utilizing this enzyme (Fig. 1). The kinetic resolutions of secondary alcohols are important processes in cases where preparation of the corresponding ketones is difficult. In these reported reactions, NAD^+ dependent enantioselective alcohol dehydrogenase (or amino acid dehydrogenase) was used with NOX for regeneration of the oxidized form of cofactor NAD^+.

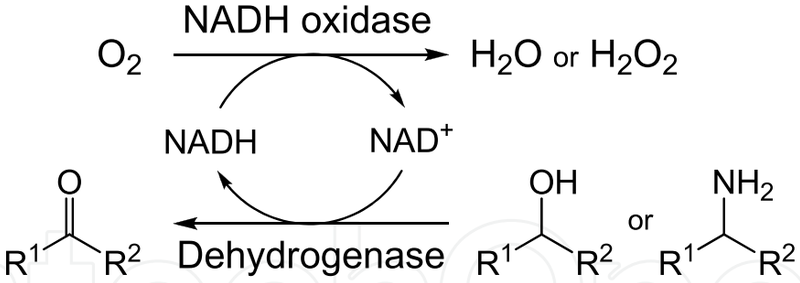

Fig. 1. Enzymatic oxidation of alcohols using a cofactor regeneration system

NADH oxidase catalyzes the oxidation of NADH to NAD^+ using molecular oxygen as the electron acceptor. The NADH oxidase family of enzymes is divided into two major types corresponding to the mode of oxygen reduction. One catalyzes a two-electron reduction of oxygen to give hydrogen peroxide, and the other catalyzes a four electron reduction of oxygen to give water with NADH oxidation. So far, NOXs have been isolated from anaerobic bacteria such as *Streptococcus* (Matsumoto et al., 1996; Higuchi et al., 1993, 1994; Ross and Claiborne, 1992; Schmidt et al., 1986), *Thermotoga* (Yang & Ma, 2005, 2007), *Clostridium* (Kawasaki et al., 2004; Maeda et al., 1992), *Eubacterium* (Herles et al., 2002), and *Lactobacillus* (Hummel et al., 2003b; Riebel et al., 2002, 2003), and from archaea such as

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Sulfolobus (Masullo et al., 1996), *Thermus* (Toomey & Mayhew, 1998; Park et al., 1992), and *Archaeoglobus* (Kengen et al., 2003). In these anaerobic organisms, NOX plays an important role as an oxygen scavenger under oxidative stress (Miyoshi et al., 2003). In aerobic microorganisms, NOX activity results from the electron transfer reaction from NADH to O_2 through cytochromes by membrane-bound enzymes. In fact, NADH oxidase of *Corynebacterium* (Matsushita et al., 2001; Ginson et al., 2000) has been isolated from the membrane. Characterization of NOX from aerobic bacteria has rarely been reported because of the difficulty in purifying the membrane proteins.

Generally, the activity of alcohol dehydrogenase in oxidation reactions is higher under basic conditions, because this reaction produces a proton. Therefore, highly active and stable NOX under basic conditions is desirable for enzymatic oxidation. We have recently isolated *Brevibacterium* sp. KU1309 from soil. This Gram-positive aerobic bacterium can grow in medium containing 2-phenylethanol as the sole source of carbon and has soluble NOX in spite of the fact that the NOXs of aerobic bacteria are generally membrane-bound enzymes. Utilizing this strain, various alcohols have been oxidized to the corresponding carboxylic acids and ketones (Miyamoto et al., 2004). Herein, we describe the isolation of a hydrogen peroxide-producing NOX from *Brevibacterium* sp. KU1309, which exhibits high thermal stability in a broad range of pH values.

2. Biocatalytic oxidation of various alcohols

The development of effective catalytic oxidation of alcohols using environmentally benign and inexpensive oxidants, such as O_2, is an important challenge (Sheldon et al., 2000). Acetic acid bacteria have been employed for the biotransformation of various alcohols (Ohta & Tetsukawa, 1979, 1981), including the enantioselective oxidation of racemic primary alcohols (Gandolfi et al., 2001; Romano et al., 2002). Whole cells are preferable as they have regeneration systems for the cofactors. A wide variety of $NAD(P)^+$-dependent dehydrogenases (EC 1.1.1.-), which catalyze the asymmetric reduction of ketones to optically active alcohols, have been reported and are used to synthesize industrially important chemicals (Nakamura et al., 2003). The application of $NAD(P)^+$-dependent enzymes to the oxidative direction is limited because few efficient methods for $NAD(P)^+$ regeneration are applicable at the preparative scale (Hummel et al., 2003a; Riebel et al., 2003). Flavin-dependent alcohol oxidases (EC 1.1.3.-) are able to oxidize alcohols to carbonyl compounds with simultaneous reduction of O_2 to H_2O_2 (Burton, 2003). Several oxidases (oxidases of methanol/ethanol, glucose, etc.) are used as analytical tools (Karube & Nomura, 2000). However, only a limited amount of information exists on the enzymatic synthesis of carbonyl compounds using alcohol oxidases. Previously, we reported the enantioselective oxidation of mandelic acid using mandelate oxidase of *Alcaligenes bronchisepticus* KU1201 (Miyamoto & Ohta, 1992; Tsuchiya et al., 1992). However, the oxidizing enzyme system was limited to mandelate derivatives. Thus, we tried to screen an oxidation system that has broad substrate specificity and high activity. We herein describe a novel method for the synthesis of carbonyl compounds via oxidation by a 2-phenylethanol-degrading microorganism.

2.1 Screening of a 2-phenylethanol-degrading microorganism

We selected 2-phenylethanol (PE, **1**) as a screening compound. Because of the toxicity of this alcohol, we expected PE-degrading microorganisms to have strong metabolic enzymes for it.

The enrichment culture technique (Asano, 2002) was used to screen for various PE-degrading microorganisms. Of the various microorganisms isolated after growing on PE, one strain was assigned to the pathway in Fig. 2. If the microorganism responsible for this oxidation reaction also acted on 2-phenylpropanol (**3**), then 2-phenylpropanoate (**4**) would be expected to be obtained because further metabolism would be impossible. Strain KU 1309 was found to be the most active in the oxidation of propanol (**3**) to propanoate (**4**). The bacterium was Gram-positive, formed rods, non-motile, non-spore-forming, and without flagella. No acid or gas was produced from glucose. The 16S rDNA sequence showed that 2-phenylethanol-utilizing strain KU 1309 is closely related to *Brevibacterium iodium* (98.2%) and *Brevibacterium epidermidis* (97.8%). Based on these results, strain KU 1309 was identified as *Brevibacterium* sp.

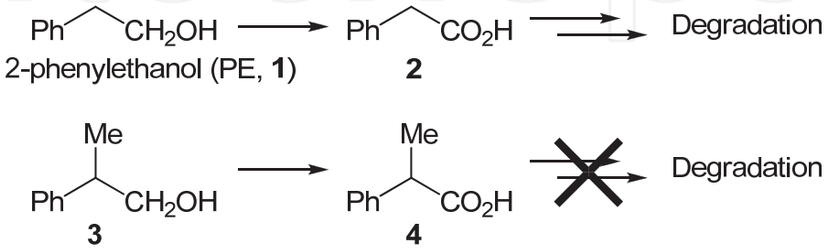


Fig. 2. 2-Phenylethanol-degrading pathway of *Brevibacterium* sp. KU1309

Although the enzyme system of the bacterium KU 1309 was demonstrated to have broad substrate specificity and high activity, it showed no enantioselectivity toward the substrate (**3**). Recently, we succeded in isolating microorganisms that oxidize 2-phenyl-1-propanol (**3**) to enantiomerically enriched (*S*)-2-phenylpropanoic acid (**4**) (Miyamoto et al., 2009). The resulting (*S*)-2-arylpropanoic acids are important compounds known as non-steroidal anti-inflammatory drugs (NSAIDs) (Kourist et al., 2011).

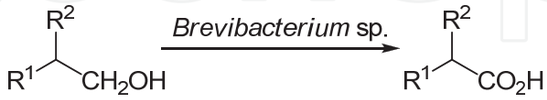
2.1.1 Optimization of microbial oxidation conditions by *Brevibacterium* sp. KU1309

The cultivation conditions suitable for inducing potent oxidative activity in intact cells were examined. The oxidation level was constant in the cells grown without PE, although the addition of alcohol increased activity. Consistent PE oxidation was obtained by using a nutrient medium containing PE. The optimum concentration of PE and the adequate cultivation period were 0.4% (w/v) and 3 d, respectively. Cell growth was inhibited by a higher PE concentration (>0.5% (w/v)). Next, the conditions for the production of acid (**4**) from alcohol (**3**) were examined. The strain exhibited highest productivity at around pH 10. When the concentration of PE was 0.1–0.4% (w/v), oxidation proceeded smoothly and the corresponding carboxylic acid was obtained in high yield (>80%). Oxidation heavily decreased when the alcohol concentration exceeded 0.6% (w/v). Therefore, the enzymatic reaction was performed at alcohol concentrations of under 0.4% (w/v).

2.1.2 Substrate specificity of microbial oxidation system

Under optimal conditions, microbial oxidation was extended to other alcohols (Table 1). First, the effect of variations in the aromatic part (R^1) was examined (Entries 1-3). These substrates were readily oxidized, and the yields of the corresponding carboxylic acids were high. The oxidizing enzyme was not enantioselective, giving racemic products. Next, the R^2 group was changed to hydrogen (Entries 4-6). Relatively high yields were realized by

quenching the reaction after 6 h. When the R^2 group was an isopropyl (Entry 7), oxidation did not proceed and the starting material was recovered intact. It is considered that the isopropyl group was too bulky for the enzyme to bind with the substrate. To investigate the cofactor requirement of the oxidizing enzyme system, we studied the effect of the reaction atmosphere. When the reaction was performed under argon, the product yield decreased drastically; thus, the oxidizing system was O_2 dependent. In addition, we tried to detect enzyme activity for the reduction of 2,6-dichloroindophenol (DCIP) at 600 nm in the presence of *N*-methylphenazonium methosulphate (PMS), or the formation of NAD(P)H at 340 nm. We detected 2-phenylethanol oxidase activity in the wall-membrane fraction and NAD^+-dependent dehydrogenase in the cytoplasm.



Entry	Substrate		Reaction time (h)	Yield (%)
	R^1	R^2		
1	Ph	Me	24	86
2	PhO	Me	24	87
3	2-Naphthyl	Me	96	80
4	Ph	H	6	70
5	$PhCH_2$	H	6	71
6	$PhCH_2CH_2$	H	6	77
7	4-Chlorophenyl	^{i}Pr	96	0

Table 1. Substrate specificity of primary alcohols

2.2 Purification and characterization of oxidation enzymes

We have shown that soil bacterium *Brevibacterium* sp. KU 1309 can be efficiently applied to the oxidation of various alcohols. The strain assimilated PE, and exhibited high oxidative activity towards various alcohols (Miyamoto et al., 2004). Endogenous 2-phenylethanol was found in the metabolic pathway of styrene in *Xantobacter* strain 124X (Hartmans et al., 1989), 2-phenylethylamine in *Escherichia coli* (Parrott et al., 1987), and phenylalanine in *Saccharomyces cerevisiae* (Dickinson et al., 2003). However, there was not sufficient information concerning the enzymes that participate in the metabolism of 2-phenylethanol, although 2-phenylethanol dehydrogenase (PEDH) was estimated to be the key enzyme of this metabolic pathway. Thus we tried to purify PEDH from *Brevibacterium* sp.

2.2.1 Purification and characterization of alcohol dehydrogenase with broad substrate specificity

The summary of the purification procedures for PEDH from *Brevibacterium* sp. is shown in Table 2 (Hirano et al., 2005). PEDH was purified about 1400-fold from the cell-free extract by sequential column chromatography.

Step	Total protein (mg)	Total activity (unit)	Specific activity (Unit/mg)	Yield (%)	Purification (fold)
Cell-free extract	1381	18.8	0.0136	100	1
$(NH_4)_2SO_4$	412	12.2	0.0296	65	2
Ether-Toyopearl	68.9	16.1	0.234	85	17
DEAE-Toyopearl	3.28	13.3	4.06	71	299
DEAE-Sepharose	1.65	7.00	4.24	37	312
Phenyl-Toyopearl	0.360	6.38	17.7	34	1302
Butyl-Toyopearl	0.198	3.80	19.2	20	1412

Table 2. Purification of PEDH from *Brevibacterium* sp. KU1309

The enzyme did not bind to Toyopearl AF-Blue and AF-Red in spite of its NAD^+ dependent oxidoreductase activity. The specific activity of PEDH increased to 19.2 U/mg of protein with a 20% yield from the cell-free extract. The purity of the enzyme was checked by SDS–PAGE, and analytical HPLC with Superose 12 gel filtration column chromatography. These analyses showed that the enzyme sample was homogenous. The molecular mass of PEDH was estimated to be 29 kDa by analytical HPLC on gel filtration column chromatography. The molecular weight under denaturing conditions was determined to be 39 kDa by SDS–PAGE. These results suggested that the purified enzyme is a monomeric protein. The N-terminal amino acid sequence of PEDH was determined to be MKASLATAIGGEFTVHD. A database search of the sequence revealed no similar proteins. Thus, the family this enzyme belongs to is very interesting. The activity of PEDH was measured at various pH values. The maximum activity of 2-phenylethanol oxidation was observed at pH 10.4. In contrast, the enzyme exhibited the maximum activity of phenylacetaldehyde reduction at pH 6.0. No significant loss of enzyme activity was observed after heat treatment for 30 min at 35°C, while about 50% of the initial activity was lost after heat treatment at 50°C. The optimal reaction temperature for the enzyme was estimated to be around 60°C. Further heat treatment above 60°C caused a rapid decrease in enzyme activity. The kinetic properties and substrate specificity fitting to Michaelis–Menten equation are shown in Tables 3–5. The kcat/Km value of NADH was about 10-fold greater than that of NAD^+ (Table 3). As a result, the reduction of aldehyde proceeded faster than the oxidation of alcohol. This enzyme did not oxidize alcohols with $NADP^+$ as cofactor: the relative reaction rate with $NADP^+$ was less than 0.1% of that with NAD^+.

cofactor	Relative activity (%)	kcat/Km (s^{-1}/mM)
NAD^+	100	22.4
$NADP^+$	<0.1	-
NADH	100	210.3
NADPH	<0.1	-

Table 3. Effect of cofactors on the relative activities

The substrate specificity of this enzyme is shown in Table 4. This enzyme oxidized various primary alcohols with aromatic rings (2-phenylethanol, 2-phenylpropanol, benzyl alcohol, 3-phenylpropanol) and primary aliphatic alcohols (ethanol, 1-butanol, 1-octanol, and 1-decanol). On the other hand, the enzyme showed lower activity toward secondary alcohols,

such as 1-phenylethanol and 2-propanol. Furthermore, this enzyme preferred (*S*)-2-phenylpropanol to the (*R*)-enantiomer as a substrate.

Substrate	Relative activity (%)	Km (mM)
2-Phenylethanol	100	0.025
(*S*)-2-Phenylpropanol	156	0.157
(*R*)-2-Phenylpropanol	63	0.020
Benzyl alcohol	199	0.012
3-Phenylpropanol	135	0.033
Ethanol	76	-
1-Butanol	111	-
1-Octanol	101	-
1-Dodecanol	68	-
1-Phenylethanol	46	-
2-Propanol	54	-

Table 4. Relative activities in the oxidation of various alcohols

Substrate	Relative activity (%)	Km (mM)
Phenylacetaldehyde	100	0.261
2-phenylpropionaldehyde	188	0.864
1-Octylaldehyde	87	-
Acetophenone	0	-

Table 5. Relative activities in the reduction of various carbonyl compounds

This enzyme reduced aldehydes with an aromatic ring (phenylacetaldehyde, 2-phenylpropionaldehyde) and an aliphatic aldehyde (1-octylaldehyde) in the reverse reaction, that is, the reduction of aldehyde. However, similar to the case of the oxidation of secondary alcohols, the enzyme could not reduce ketones (acetophenone) (Table 5). Various compounds were investigated for their effects on enzyme activity. The enzyme activity was completely inhibited by 1 mM of $CuCl_2$ (0%), $NiCl_2$ (7%), $BaCl_2$ (5%), and $HgCl_2$ (0%). Other inorganic compounds, such as $MgCl_2$, $CaCl_2$, $MnCl_2$, and $ZnCl_2$ had no influence on activity. While the enzyme was completely inhibited by *p*-chloromercuribenzoate, other thiol reagents such as iodoacetate and N-ethylmaleimide did not have inhibitory effects. Metal chelating reagents, such as 8-quinolinol (98%) and EDTA (99%), carbonyl reagents such as NaN_3 (94%), and serine inhibitors, for example, phenylsulfonyl fluoride (85%), had no significant effect on the enzyme. We isolated PEDH from 2-phenylethanol-assimilating soil bacterium *Brevibacterium* sp. which was grown in medium containing 2-phenylethanol as the sole source of carbon. PEDH oxidized 2-phenylethanol to phenylacetaldehyde, and utilized NAD^+ but not $NADP^+$ as the cofactor. Previously, we detected 2-phenylethanol oxidase activity in the wall-membrane fraction (Miyamoto et al., 2004). Phenylacetaldehyde dehydrogenase (PADH) activity was also recognized in the cell-free extracts of the strain. The present results suggest that the oxidative degradation of 2-phenylethanol (**1**) via phenylacetaldehyde (**5**) to phenylacetate (**2**) is one of

the metabolic pathways of 2-phenylethanol by *Brevibacterium* sp. PEDH plays a role, in part, in the oxidation of 2-phenylethanol into phenylacetaldehyde in this strain (Fig. 3). The substrate specificity of PEDH is different from that of NAD^+-dependent aromatic alcohol dehydrogenase. Aryl alcohol dehydrogenase (EC 1.1.1.90) from *Pseudomonas putida* could catalyze the oxidation of benzyl alcohol but not 2-phenylethanol and alkanol (Shaw & Harayama, 1990). Benzyl alcohol dehydrogenase from benzyl alcohol-assimilating *Thauera* sp. similarly could not oxidize alkanol (Biegert et al., 1995). Although phenylacetaldehyde reductase from styrene-assimilating *Corynebacterium sp.* strain ST-10 reduced various aldehydes, it did not catalyze the reverse reaction (Itoh et al., 1996). The dehydrogenase of *P. putida* appears as a homodimer composed of a 42 kDa subunit. Alcohol dehydrogenases (ADHs) from *Thauera* sp. and *Corynebacterium* sp. display similar subunit sizes (40–42 kDa) and were found to be homotetramers. The active form of PEDH is a monomer, while the size (39 kDa) of PEDH is similar to those of the ADHs listed above. The alcohol dehydrogenase superfamily can be divided into three groups: group I is the zinc-dependent long chain ADHs (approximately 350 residues per subunit); group II is the short chain zinc-independent ADHs (approximately 250 residues per subunit); and group III is the iron-activated ADHs (approximately 385 residues per subunit) (Reid & Fewson, 1994). From the results of inhibition studies, PEDH is a zinc-independent alcohol dehydrogenase containing approximately 350 residues per subunit. Thus, PEDH does not fit into any groups. Furthermore, the N-terminal amino acid sequence of PEDH showed no similarity to other reported dehydrogenases. Based on these results, it can be said that PEDH is a novel enzyme in terms of these characteristic properties and its broad substrate specificity. Thus, PEDH from *Brevibacterium* sp., in combination with an NAD^+ regeneration system, would be a new entry of a clean and versatile alcohol oxidizing system.

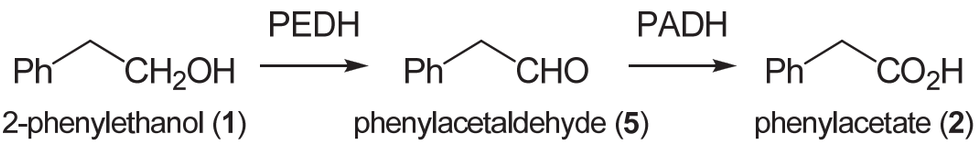


Fig. 3. Estimated metabolic pathway of 2-phenylethanol by *Brevibacterium* sp.

2.2.2 Purification and characterization of the aldehyde dehydrogenase

First, the localization of phenylacetaldehyde dehydrogenase (PADH) in the cell was examined (Hirano et al., 2007). The cell-free extract, which had PADH activity, was fractionated by ultracentrifugation (105,000×g, 60 min). NAD^+-dependent phenylacetaldehyde-oxidizing activity was found in the supernatant fraction, indicating that PADH is a cytoplasmic enzyme. The summary of the purification procedure for PADH from *Brevibacterium* sp. KU1309 is shown in Table 6. The specific activity of the enzyme increased by 16-fold to 4.16 U/mg protein with a 0.21% yield from the cell-free extract. The low yield of purified PADH through this procedure is considered to be due to the low stability of the enzyme. The molecular weight of the denatured protein was estimated to be approximately 61 kDa based on a comparison with the mobility of the marker proteins. The molecular mass of native PADH was estimated to be 219 kDa by gel filtration on high-performance liquid chromatography. These results indicate that the enzyme is a homotetrameric protein. Because the reported PadA from *E. coli* was a homodimer, PADH is obviously different from this enzyme.

Step	Total protein (mg)	Total activity (unit)	Specific activity (Unit/mg)	Yield (%)	Purification (fold)
Cell-free extract	480	125	0.261	100	1
Butyl-Toyopearl	6.15	2.01	0.327	1.6	1.25
DEAE-Toyopearl	0.620	0.864	1.39	0.69	5.34
DEAE-Sepharose	0.132	0.250	1.89	0.20	7.26
Ether-Toyopearl	0.063	0.262	4.16	0.21	15.9

Table 6. Purification of PADH from *Brevibacterium* sp. KU1309

The N-terminal sequence of PADH was found to be TTTVESPARSP. A database search for protein sequences resembling this protein was conducted using BLAST or FASTA systems. However, no protein with similar aldehyde dehydrogenase activity was found. The optimum pH and pH stability of PADH were determined. PADH exhibited oxidizing activity at pH 7-10, with the maximum activity observed at pH 9. The pH stability was examined by measuring activity at pH 9 after incubation for 30 min at various levels of pH. This enzyme was relatively stable between pH 6 and 8. At pH 8.0, PADH retained 60% of its initial activity. PADH showed highest activity at 35°C. At this temperature, residual activity was 40% after 30 min. The effects of various compounds on the activity of PADH were determined. PADH was activated upon incubation in the presence of several divalent cations such as Mg^{2+} (142%), Ca^{2+} (153%), and Mn^{2+} (117%). A similar effect was reported for ALDH1 from *Saccharomyces cerevisiae* (Wang et al., 1998). Enzyme activity was inhibited by sulfhydryl agents such as Hg^{2+} (23%), *p*-CMBA (0%), iodoacetamide (0%), and N-methylmaleimide (0%), which indicates that a thiol group plays an important role in oxidation. PADH was not stimulated by potassium ions, unlike ALDH2 from *S. cerevisiae* and BADH from *Pseudomonas putida* (McLeish et al., 2003). PADH activity was inhibited by a high concentration (0.1 mM) of phenylacetaldehyde. This property is the same as that for PadA from *E. coli*. The kinetic parameters of PADH were determined by Lineweaver–Burk plotting (Table 7). The Km of PADH for phenylacetaldehyde was low (1.24 μM), and substrate inhibition was observed even at low substrate concentrations. The low Km value is on the same order as the Km of PAD from *E. coli* K12 (7 μM; Hanlon et al., 1997). This result suggested that phenylacetaldehyde is probably the natural substrate for PADH. On the other hand, the Km of PADH for NAD^+ (116 μM) is greater than that of phenylacetaldehyde dehydrogenase from *Achromobacter eurydice* (70 μM). The substrate specificity of PADH was investigated using a variety of aldehydes (Table 8). PADH oxidized a wide range of aldehydes, such as aromatic aldehydes (2-phenylethanol, benzylalcohol, 3-phenylpropanol, and 1-naphthaldehyde) and aliphatic aldehydes (hexanal, octanal, and decanal). The enzyme showed highest activity toward phenylacetaldehyde, and the activity for octylaldehyde was highest. This enzyme prefers NAD^+ as the cofactor rather than $NADP^+$ (6%).

Substrate	Km (μM)	Vmax (μmol min^{-1} mg^{-1})
Phenylacetaldehyde	1.24	4.63
NAD^+	116	4.40

Table 7. Kinetic parameters for PADH

Substrate	Relative activity (%)
Phenylacetaldehyde	100
Benzaldehyde	54
3-Phenylpropionaldehyde	34
1-Naphthaldehyde	24
Hexanal	21
Octanal	31
Decanal	17
NAD^+	100
$NADP^+$	6

Table 8. Substrate specificity of PADH

We have isolated phenylacetaldehyde dehydrogenase from *Brevibacterium* sp. KU1309 as the key enzyme in the 2-phenylethanol metabolic pathway. The anticipated catabolic pathway of 2-phenylethanol is shown in Fig. 4; *Brevibacterium* sp. KU1309 oxidizes 2-phenylethanol to phenylacetaldehyde by NAD^+-dependent PEDH. As PEDH could not oxidize aldehyde (data not shown), the resulting phenylacetaldehyde is oxidized to phenylacetate by NAD^+-dependent PADH. Phenylacetate should be further degraded via the general pathway of aromatic compounds. To the best of our knowledge, the metabolic pathway of 2-phenylethanol via phenylacetate from 2-phenylethanol-assimilating bacteria has not been reported.

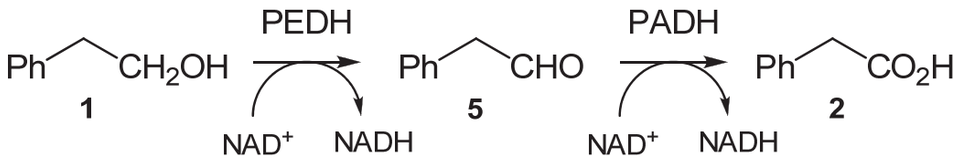


Fig. 4. The metabolic pathway of 2-phenylethanol in *Brevibacterium* sp.

As mentioned earlier, the intermediate of the metabolic pathway of phenylalanine, styrene, and 2-phenylethylamine is also phenylacetaldehyde. However, the N-terminal amino acid sequence of purified PADH in the present work was not similar to the sequences of the other phenylacetaldehyde dehydrogenases. Thus, a genetic comparison of the gene encoding PADH with those of the corresponding aldehyde dehydrogenases of the other three pathways is of great interest and the target of future studies. The property of PADH is also different from other aldehyde dehydrogenases relating to phenylacetaldehyde. For example, PadA, which originates from *E. coli* w3110 and is a homodimeric enzyme, could not oxidize benzaldehyde. MdlD from *Pseoudomonas putida*, which has a broad substrate range of aryl or aliphatic aldehydes, is a K^+-activated enzyme. However, PADH is a homotetrameric enzyme and activated by divalent metal ions. Moreover, PADH also oxidizes benzaldehyde and aliphatic aldehydes. PADH has broad substrate specificity and would be extremely useful as an aldehyde-oxidizing biocatalyst such as commercially available ALDH originating from *S. serevisiae*. We isolated and characterized PADH from *Brevibacterium* sp. KU1309. The enzyme has broad substrate specificity and a different N-terminal sequence from those of known aldehyde dehydrogenases

2.2.3 Purification and characterization of thermostable H_2O_2-forming NADH oxidase

A summary of the purification of NADH oxidase (BreNOX) from *Brevibacterium* sp. is shown in Table 9 (Hirano et al., 2008). About one third of NOX activity was detected in the cytoplasmic fraction, and the remaining 60% was detected in the membrane fraction after ultracentrifugation (105,000 ×g, 60 min). We purified BreNOX from the cytoplasmic fraction. BreNOX was purified to homogeneity by ammonium sulfate fractionation and three steps of column chromatography.

Step	Total protein (mg)	Total activity (unit)	Specific activity (Unit/mg)	Yield (%)	Purification (fold)
Cell-free extract	800.9	69.1	0.086	100	1
$(NH_4)_2SO_4$	466.7	33.3	0.071	48	0.83
Phenyl-Toyopearl	16.55	15.7	0.94	23	10.9
DEAE-Toyopearl	2.31	9.73	4.2	14	48.8
Butyl-Toyopearl	1.35	7.04	5.2	10	60.5

Table 9. Purification of BreNOX

Purified BreNOX showed a single band on SDS/PAGE stained with Coomassie brilliant blue. The molecular weight was found to be about 57 kDa. The molecular weight of native BreNOX was estimated to be about 102 kDa by gel filtration. These results show that the native enzyme has a homodimeric structure. The enzyme shows a yellow color, which is probably because of the binding of FAD as the cofactor. NADH oxidases can be divided into two major categories: one group produces H_2O_2 from O_2 and the other produces H_2O from O_2. The reduction of molecular oxygen was examined to clarify the category in which the present enzyme belongs. Oxidation of NADH catalyzed by this enzyme resulted in the stoichiometric production of H_2O_2, which was confirmed by the spectrophotometrical method (Kengen et al., 2003), i.e., detection of a dye compound formed by electron transfer from H_2O_2 using peroxidase. Thus, it was found that BreNOX belongs to the H_2O_2-producing type. The effects of various salts on the activity of BreNOX were examined (Table 10). The activity of the enzyme increased about ten-fold with the addition of ammonium sulfate and ammonium chloride (Fig. 5). On the other hand, sodium chloride and potassium chloride had no influence on NOX activity. Accordingly, the activation of this enzyme upon addition of the two salts would be due to the ammonium ion. Although the precise mechanism is not clear, it is supposed that the ammonium cation may bind to some anionic part of the enzyme resulting in a change of conformation.

Salts	Relative activity (%)
None	100
$(NH_4)_2SO_4$	1000
NH_4Cl	940
NaCl	80
KCl	100

Table 10. Effect of salts on NOX activity

The optimal reaction temperature for the enzyme was determined to be 70°C, and the activity at 30°C was about 40% of the maximal. As shown in Fig. 6, no significant loss of enzyme activity was observed after heat treatment at 70°C for 60 min, while about 30% of the initial activity was lost after incubation at 80°C for 60 min. Thus, it can be said that BreNOX is a thermostable enzyme. To the best of our knowledge, such a thermally stable enzyme has not been reported except for the NADH oxidase from hyperthermophilic archaeon *Archaeoglobus fulgidus*. It is very interesting that the enzyme from *Brevibacterium* is stable at high temperature regardless of the fact that this strain grows at normal temperatures under aerobic conditions.

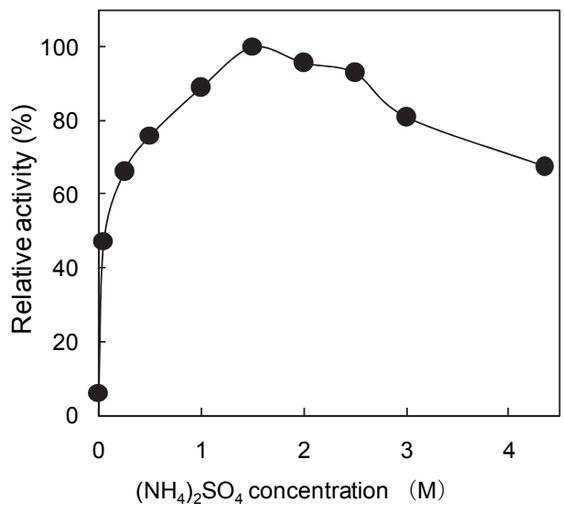


Fig. 5. The effect of ammonium sulphate concentration

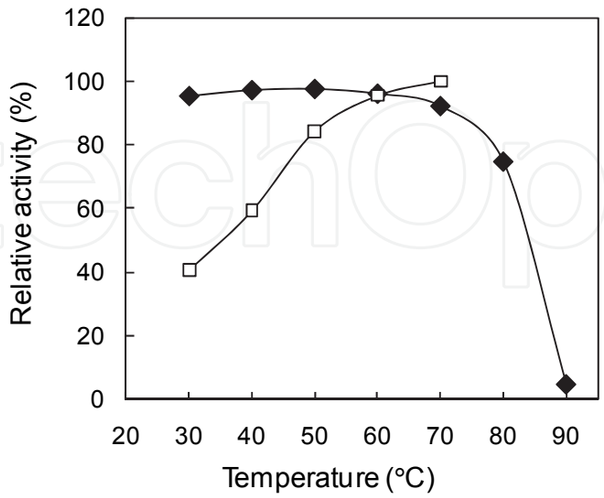


Fig. 6. The effect of temperature on activity and stability. Remaining activity after incubation for 1 h at various temperatures (closed diamond). Relative activity at various temperatures (open square)

The effect of pH on the activity and stability of BreNOX was examined. The enzyme was active and stable in the pH range 6–10 (Figs. 7 and 8), while maximal activity was observed at pH 9. These results indicated that the enzyme is an alkalophilic enzyme.

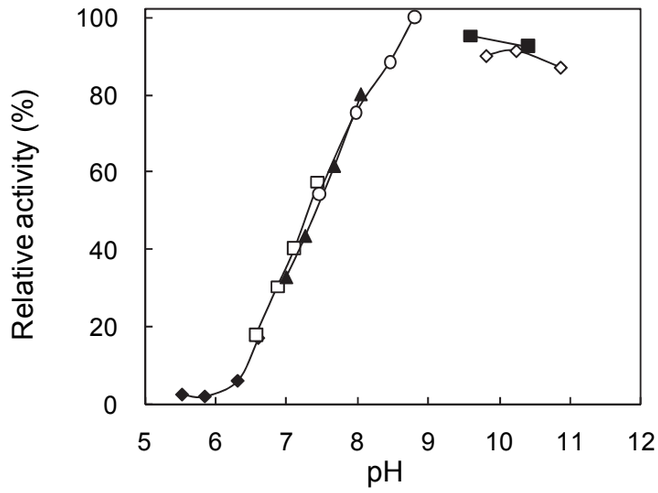


Fig. 7. The effect of pH on NOX activity. The activity of BreNOX was measured at various pH levels. The buffers used were MES (pH 5.5-6.6; closed diamond), MOPS (pH 6.6-7.4; open square), HEPES (pH 7.0-8.1; closed triangle), Tris (pH 7.5-8.8; open circle), Glycine (pH 9.6-10.4; closed square), and CAPS (pH 9.8-10.9; open diamond)

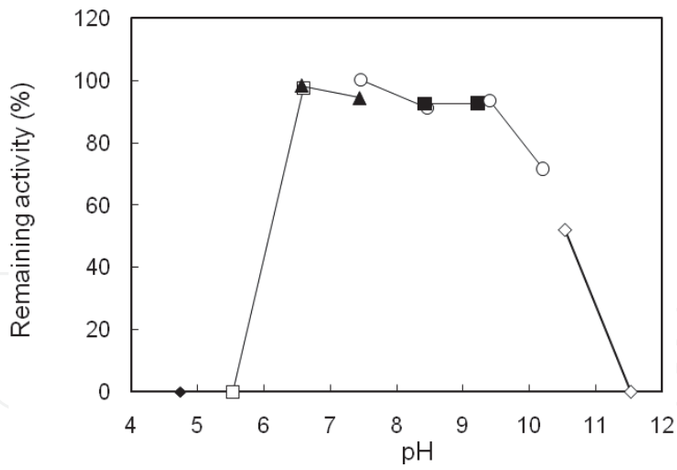


Fig. 8. The effect of pH on stability. The remaining activity of BreNOX was measured after incubation for 1 h at various pH levels. The buffers were citric acid (pH 4.7; closed diamond), MES (pH 5.5-6.6; open square), MOPS (pH 6.6-7.4; closed triangle), Tris (pH 7.5-8.5; open circle), TAPS (pH 8.4-9.2; closed square), CAPS (pH 9.4–10.2; open circle), and Na_2HPO_4 (pH 10.5–11.5; open diamond)

The effects of various compounds on enzyme activity were investigated. NOX activity was inhibited by the presence of 1 mM of some metal ions, such as Zn^{2+} (39%), Cu^{2+} (43%), Hg^{2+} (71%), and Ag^{+} (37%). Curiously, enzyme activity was nearly doubled by adding *p*-CMBA.

Cofactors such as FAD and FMN had no effect on activity. The Km and Vmax values of NOX were found to be 0.022 mM and 8.86 U/mg, respectively, in the presence of 500 mM ammonium sulfate. The Km value of the enzyme was similar to that of other types of NOX. In addition, the enzyme showed little activity toward the oxidation of NADPH. The N-terminal amino acid sequence was found to be XDELTYDLVVLGGGTGG. A FASTA-based search of a protein database indicated that this sequence exhibited high identity with those of the pyridine nucleotide–disulfide oxidoreductase family (dihydrolipoamide dehydrogenase and glutathione reductase). Because the N-terminal amino acid residue could not be determined by Edman degradation, it is estimated that the amino group of this amino acid residue is protected.

2.3 Coupling reaction with dehydrogenases and BreNOX

Recycling of the cofactor is the key process for effective oxidative biotransformation. If the newly discovered BreNOX can be applied to cofactor regeneration of oxidative reactions that require NAD^+, the whole system will be extremely useful for the green process. Thus, we tried the coupled reaction as demonstrated below (Fig. 9). First, BreNOX was coupled with mandelate dehydrogenase (MDH) for cofactor regeneration (eq. 1). MDH from *E. faecalis* (Tamura et al., 2002) oxidized (*R*)-mandelic acid (**6**) using NAD^+ as the electron acceptor. The enantioselective oxidation of racemic mandelic acid using MDH and NOX proceeded smoothly. The (*R*)-mandelic acid was oxidized to benzoylformate (**7**), and the recovered mandelic acid was found to be *S* configuration with over 99% e.e. The result showed that (*R*)-mandelate was oxidized completely in the presence of only 0.05 mol equivalent of NAD^+. L-phenylalanine (**8**) was completely oxidized to phenylpyruvate (**9**) in the pure D-phenylalanine (99% e.e.) in a coupled reaction with L-phenylalanine dehydrogenase (LPADH) (eq. 2) using racemic phenylalanine.

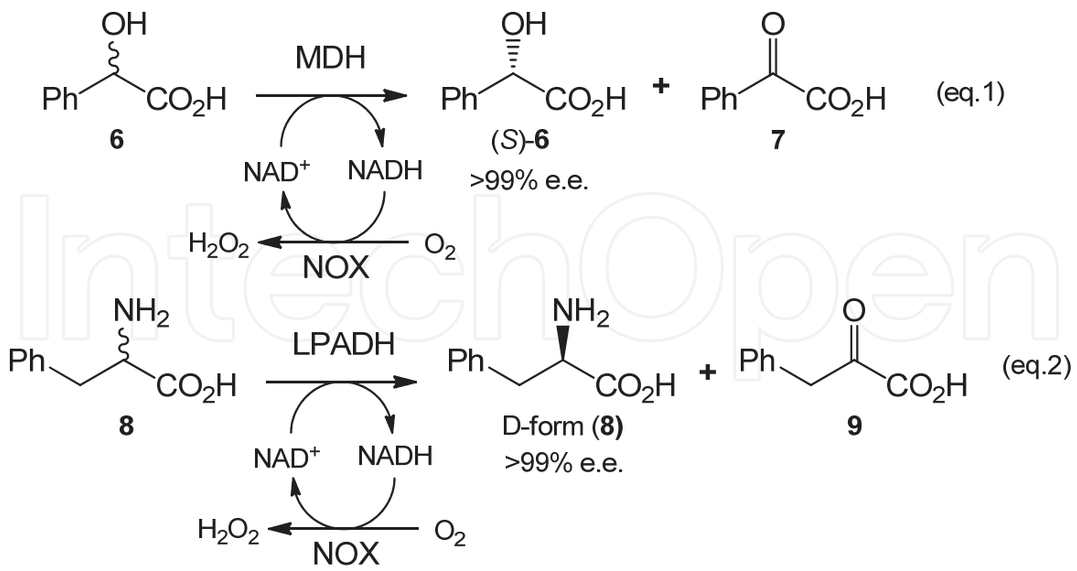
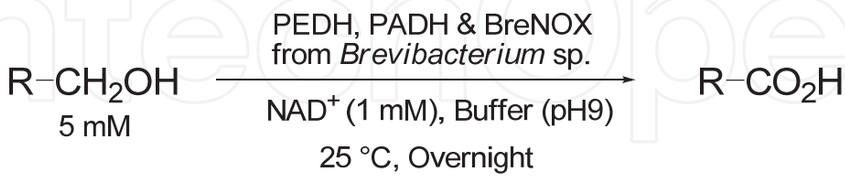


Fig. 9. Coupling reaction with dehydrogenase and NOX

Next, BreNOX was coupled with 2-phenylethanol dehydrogenase (PEDH) and phenylacetaldehyde dehydrogenase (PADH) from *Brevibacterium* sp. (Table 11). The system

for the coupled reaction was composed of 5 mM of various alcohols, 1 mM of NAD^+, and Tris–HCl buffer (100 mM, pH 8.8). The mixture was incubated overnight at 25°C. Hydrochloric acid (2 M) was added to the reaction mixture, and the mixture was extracted with diethyl ether. After esterification by $TMSCH_2N_2$, the esters were analyzed by GLC to determine the yield. The oxidation products were obtained quantitatively. As demonstrated by the above two examples, BreNOX can be successfully coupled with various NAD^+-dependent enzymes, because of high activity under a wide range of reaction conditions.



Entry	Substrate	Carboxylic acid (%)	Alcohol (%)
1	$PhCH_2OH$	74	2
2	$PhCH_2CH_2OH$	86	3
3	$PhCH_2CH_2CH_2OH$	87	0
4	$CH_3(CH_2)_4CH_2OH$	60	5
5	$CH_3(CH_2)_6CH_2OH$	57	3

Table 11. Oxidation of primary alcohols using PEDH, PADH and BreNOX

3. Conclusion

We have succeeded in isolating a hydrogen peroxide producing NOX from the aerobic bacterium *Brevibacterium* sp. KU1309, which showed activity and stability in a broad range of pH. The alignment of N-terminal amino acid sequence is shown in Fig. 10.

```
Brevibacterium sp. KU1309                -------XDE LTYDLVVLGG GTGG
Bacillus sp. NRRL B-14911                --------MA QEYDLVILGG GTGG
Limnobacterium sp. MED105                -----MSTPN APYDLVVIGG GSGG
Mycobacterium smegmatis                  MS-HPGATAS DRHKVVIIGS GFGG
Psedomonas fluorescens                   -------MT- --HRIVIVGG GAGG
Corynebacterium glutamicum ATCC13032     MSVNPTRPEG GRHHVVVIGS GFGG
Escherichia coli                         -------MTT PLKKIVIVGG GAGG
```

Fig. 10. Alignment of the N-terminal amino acid sequence of various flavoproteins from bacteria. *Brevibacterium* sp. KU1309; Dihydrolipoamide dehydrogenase (AC: Q2B5N0) from *Bacillus* sp. NRRL B-14911; Glutathione reductase (AC: A6GLK6) from *Limnobacter* sp. MED105; NADH dehydrogenase (AC: A0QYD6) from *Mycobacterium smegmatis*; NADH dehydrogenase (AC: Q9KGX0) from *Pseudomonas fluorescens*; NADH dehydrogenase (AC: Q79VG1) from *Corynebacterium glutamicum* ATCC13032; and NADH dehydrogenase (AC: Q0TIW5) from *Escherichia coli*. Italic letters represent the consensus hydrophobic region. Bold letters represent the binding sites for FAD or NAD.

The sequence of dihydrolipoamide dehydrogenase from *Bacillus* sp. shows the highest similarity to the proteins originating from this aerobic bacterium. The enzyme from *Limnobacterium* sp. was a glutathione reductase. The other four enzymes were NADH dehydrogenases. These enzymes have characteristic consensus sequences in the N-terminal region, which is the hydrophobic and glycine rich region, such as GXGXXG for binding NADH or FAD. Accordingly, the NOX obtained in this study can be regarded as belonging to the FAD-dependent pyridine nucleotide reductase family. However, while the enzyme has NADH-oxidizing activity, the sequence of the N-terminus is more like that of dihydrolipoamide dehydrogenases than that of NADH oxidases. Therefore, BreNOX may have other catalytic activities because homologous enzymes of this family exhibit various activities, such as those related to peroxidases and disulfide reductases. The cloning and analysis of the sequence of BreNOX is an interesting future subject. It was found that BreNOX had properties characteristic of the dihydrolipoamide dehydrogenase family. Although a NOX has been isolated from the membrane fraction of an aerobic bacterium, such as NOX from *Corynebacterium glutamicum* (Matsushita et al., 2001), the NOX in this study was found in the cytoplasm. Moreover, to the best of our knowledge, this is the first observation of the activation of NOX by ammonium ions. It is estimated that a change in the secondary or tertiary structure of the enzyme due to high ionic strength, as well as the effect of pH, might bring about an increase in the activity of the enzyme. In addition, the pH profile of this enzyme characteristically shows maximum activity under alkaline conditions, whereas other members of the NOX family are most active under neutral or acidic pH conditions (Table 12).

Organisms	M.W. (kDa)	Km (μM)	Specific activity (U/mg)	Product of O_2 reduction	Optimal pH
Aerobic bacteria					
Brevibacterium sp.	57, α2	22	8.86 (25°C)	H_2O_2	9
C. glutamicum	55	n.d.	46.5 (25°C)	n.d.	6.5
Obligate anaerobic bacteria					
T. hypogea	50, α2	7.5	37 (80°C)	H_2O_2	7
C. aminovalericum	45, α2	19.2	130 (37°C)	H_2O	7
Thermophilic anaerobic archaea					
A.flugidus NOXA-1	48	130	8.7 (80°C)	H_2O_2	8
A.flugidus NOXB-1	61	11	1.5 (70°C)	H_2O_2	6.5

Table 12. Comparison of the properties of NADH oxidases

Moreover, this enzyme exhibits high activity in a broader range of temperatures and high thermal stability compared with NOX enzymes from thermophilic bacteria and archaea. Although NOX enzymes obtained from thermophilic archaea have high thermal stability, they show low or no activity at room temperature. On the other hand, because BreNOX exhibits high activity even at lower temperatures, in addition to thermal stability, it would be more applicable to coupled reactions with various dehydrogenases. In conclusion, we have purified and characterized NADH oxidase (BreNOX) from *Brevibacterium* sp. KU1309.

Although the physiological roles of the enzyme have not been fully disclosed, enzymatic properties such as stability and responses to salt and pH have been characterized and are considered to be useful for organic synthesis. Problems in cloning and over-expression of the gene will need to be overcome to raise the efficiency of the enzyme.

4. References

Asano, Y. (2002). Overview of screening for new microbial catalysts and their uses in organic synthesis – selection and optimization of biocatalysts. *Journal of Biotechnology*, Vol.94, No.1, pp. 65-72, ISSN 0168-1656

Biegert, T.; Altenschmidt, U.; Eckerskorn, C. & Fuchs, G. (1995). Purification and properties of benzyl alcohol dehydrogenase from a denitrifying *Thauera* sp. *Archives of Microbiology*, Vol.163, No.6, pp. 418-423, ISSN 0302-8933

Burton, S.G. (2003). Oxidizing enzymes as biocatalysts. *Trends in Biotechnology*, Vol.21, No.12, pp. 543-549, ISSN 0167-7799

Dickinson, J. R.; Salgado, L. E. J. & Hewlins, M. J. E. (2003). The catabolism of amino acids to long chain and complex alcohols in *Saccharomyces cerevisiae*. *Journal of Biological Chemistry*, Vol.278, No.10, pp. 8028-8034, ISSN 0021-9258

Gandolfi, R.; Ferrara, N. & Molinari, F. (2001). An easy and efficient method for the production of carboxylic acids and aldehydes by microbial oxidation of primary alcohols. *Tetrahedron Letters*, Vol.42, No.3, pp. 513-514, ISSN 0040-4039

Geueke, B.; Riebel, B. & Hummel, W. (2003). NADH oxidase from *Lactobacillus brevis*: a new catalyst for the regeneration of NAD. *Enzyme and Microbial Technology*, Vol.32, No.2, pp. 205-211, ISSN 0141-0229

Ginson, C.M.; Mallett, T.C.; Claiborne, A.I. & Caparon, M.G. (2000). Contribution of NADH oxidase to aerobic metabolism of *Streptococcus pyogenes*. *Journal of Bacteriology*, Vol.182, No.2, pp. 448-455, ISSN 0021-9193

Hanlon, S.P.; Hill, T.K.; Flavell, M.A.; Joseph, M.; Stringfellow, J.M. & Cooper, R.A. (1997). 2-Phenylethylamine catabolism by *Escherichia coli* K-12: gene organization and expression. *Microbiology*, Vol.143, No.2, pp. 513-518, ISSN 1350-0872

Hartmans, S.; Smits, J. P.; Van der Werf, M. J.; Volkering, F. & De Bont, J. A.M. (1989). Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. *Applied and Environmental Microbiology*, Vol.55, No.11, pp. 2850-2855, ISSN 0099-2240

Herles, C.; Braune, A. & Blaut, M. (2002). Purification and characterization of an NADH oxidase from *Eubacterium ramulus*. *Archives of Microbiology*, Vol.178, No.1, pp. 71-74, ISSN 0302-8933

Higuchi, M.; Shimada, M.; Matsumoto, J.; Yamamoto, Y.; Rhaman, A. & Kamio, Y. (1994). Molecular cloning and sequence analysis of the gene encoding the H_2O_2-forming NADH oxidase from *Streptococcus* mutans. *Bioscience, Biotechnology, and Biochemistry*, Vol. 58, No.9, pp. 1603-1607, ISSN 0916-8451

Higuchi, M.; Shimada, M.; Yamamoto, Y.; Hayashi, T.; Koga, T. & Kamio, Y. (1993). Identification of two distinct NADH oxidases corresponding to H_2O_2-forming oxidase and H_2O-forming oxidase induced in *Streptococcus* mutans. *Journal of General Microbiology*, Vol.139, No.10, pp. 2343-2351, ISSN 0022-1287

Hirano, J.; Miyamoto, K. & Ohta, H. (2005). Purification and characterization of the alcohol dehydrogenase with a broad substrate specificity originated from 2-phenylethanol-assimilating *Brevibacterium* sp. KU 1309. *Journal of Bioscience and Bioengineering*, Vol.100, No.3, pp. 318-322, ISSN 1389-1723

Hirano, J.; Miyamoto, K. & Ohta, H. (2007). Purification and characterization of aldehyde dehydrogenase with a broad substrate specificity originated from 2-phenylethanol-assimilating *Brevibacterium* sp. KU1309. *Applied Microbiology and Biotechnology*, Vol.76, No.2, pp. 357-363, ISSN 0175-7598

Hirano, J.; Miyamoto, K. & Ohta, H. (2008). Purification and characterization of thermostable H_2O_2-forming NADH oxidase from 2-phenylethanol-assimilating *Brevibacterium* sp. KU1309. *Applied Microbiology and Biotechnology*, Vol.80, No.1, pp. 71-78, ISSN 0175-7598

Hummel, W. & Riebel, B. (1996). Chiral alcohols by enantioselective enzymatic oxidation. *Annals of the New York Academy of Sciences*, Vol.799 (Enzyme Engineering XIII), pp. 713-716, ISSN 0077-8923

Hummel, W.; Kuzu, M. & Geueke, B. (2003a). An efficient and selective enzymatic oxidation system for the synthesis of enantiomerically pure D-tert-leucine. *Organic Letters*, Vol.5, No.20, pp. 3649-3650, ISSN 1523-7060

Hummel, W. & Riebel, B. (2003b). Isolation and biochemical characterization of a new NADH oxidase from *Lactobacillus brevis*. *Biotechnology Letters*, Vol.25, No.1, pp. 51-54, ISSN 0141-5492

Itoh, N.; Yoshida, K. & Okada, K. (1996). Isolation and identification of styrene-degrading *Corynebacterium* strains, and their styrene metabolism. *Bioscience, Biotechnology, and Biochemistry*, Vol.60, No.11, pp. 1826-1830, ISSN 0916-8451

Karube, I. & Nomura, Y. (2000). Enzyme sensors for environmental analysis. *Journal of Molecular Catalysis B: Enzymatic*, Vol.10, No.1-3, pp. 177-181, ISSN 1381-1177

Kawasaki, S.; Ishikura, J.; Chiba, D.; Nishino, T. & Niimura, Y. (2004). Purification and characterization of an H_2O-forming NADH oxidase from *Clostridium aminovalericum*: existence of an oxygen-detoxifying enzyme in an obligate anaerobic bacteria. *Archives of Microbiology*, Vol.181, No.4, pp. 324-330, ISSN 0302-8933

Kengen, S.W.; van der Oost, J. & de Vos, W.M. (2003). Molecular characterization of H_2O_2-forming NADH oxidases from *Archaeoglobus fulgidus*. *European Journal of Biochemistry*, Vol.270, No.13, pp. 2885-2894, ISSN 0014-2956

Kourist, R.; Domínguez de María, P. & Miyamoto, K. (2011). Biocatalytic strategies for the asymmetric synthesis of profens – recent trends and developments. *Green Chemistry*, Vol.13, No.10, pp. 2607-2618, ISSN 1463-9262

Maeda, K.; Truscott, K.; Liu, X.L. & Scopes, R.K. (1992). A thermostable NADH oxidase from anaerobic extreme thermophiles. *Biochemical Journal*, Vol.284, No.2, pp. 551-555, ISSN 0306-3275

Masullo, M.; Raimo, G.; Dello Russo, A.; Bocchini, V. & Bannister, J.V. (1996). Purification and characterization of NADH oxidase from the archaea *Sulfolobus acidocaldarius* and *Sulfolobus solfataricus*. *Biotechnology and Applied Biochemistry*, Vol.23, No.1, pp. 47-54, ISSN 0885-4513

Matsumoto, J.; Higuchi, M.; Shimada, M.; Yamamoto, Y. & Kamio, Y. (1996). Molecular cloning and sequence analysis of the gene encoding the H_2O-forming NADH oxidase from *Streptococcus* mutans. *Bioscience, Biotechnology, and Biochemistry*, Vol.60, No.1, pp. 39-43, ISSN 0916-8451

Matsushita, K.; Otofuji, A.; Iwahashi, M.; Toyama, H. & Adachi, O. (2001). NADH dehydrogenase of *Corynebacterium glutamicum*. Purification of an NADH dehydrogenase II homolog able to oxidize NADPH. *FEMS Microbiology Letters*, Vol.204, No.2, pp. 271-276, ISSN 0378-1097

McLeish, M.J.; Kneen, M.M.; Gopalakrishna, K.N.; Koo, C.W.; Babbitt, P.C.; Gerlt, J.A. & Kenyon, G.L. (2003). Identification and characterization of a mandelamide hydrolase and an NAD(P)$^+$-dependent benzaldehyde dehydrogenase from *Pseudomonas putida* ATCC 12633. *Journal of Bacteriology*, Vol.185, No.8, pp. 2451-2456, ISSN 0021-9193

Miyamoto, K. & Ohta, H. (1992). Enantioselective oxidation of mandelic acid using a phenylmalonate metabolizing pathway of a soil bacterium *Alcaligenes bronchisepticus* KU 1201. *Biotechnology Letters*, Vol.14, No.5, pp. 363-366, ISSN 0141-5492

Miyamoto, K.; Hirano, J. & Ohta, H. (2004). Efficient oxidation of alcohols by a 2-phenylethanol-degrading *Brevibacterium* sp. *Biotechnology Letters*, Vol.26, No.17, pp. 1385-1388, ISSN 0141-5492

Miyamoto, K.; Fujimori, K.; Hirano, J. & Ohta, H., (2009). Microbial kinetic resolution of 2-substituted-1-propanol, *Biocatalysis and Biotransformation*, Vol.27, No.1, pp. 66-70, ISSN 1024-2422

Miyoshi, A.; Rochat, T.; Gratadoux, J.J.; Le Loir, Y.; Oliveira, S.C.; Langella, P.& Azevedo, V. (2003). Oxidative stress in *Lactococcus lactis*. *Genetics and Molecular Research*, Vol.2, No.4, pp. 348-359, ISSN 1676-5680

Nakamura, K.; Yamanaka, R.; Matsuda, T. & Harada, T. (2003). Recent development in asymmetric reduction of ketones with biocatalysts. *Tetrahedron: Asymmetry*, Vol.14, No.18, pp. 2659-2681, ISSN 0957-4166

Ohta, H. & Tetsukawa, H. (1979). Enantiotopically selective oxidation of 1,3-diols with microorganism. *Chemistry Letters*, No.11, pp. 1379-1380, ISSN 0366-7022

Ohta, H. & Tetsukawa, H. (1981). Enantiotopically selective oxidation of 1,5-diols with *Gluconobacters* preparation of (*S*)-mevalonolactone. *Agricultural and Biological Chemistry*, Vol.45, No.8, pp. 1895-1896, ISSN 0002-1369

Park, H.J.; Reiser, C.O.; Kondruweit, S.; Erdmann, H.; Schmid, R.D. & Sprinzl, M. (1992). Purification and characterization of a NADH oxidase from the thermophile *Thermus thermophilus* HB8. *European Journal of Biochemistry*, Vol.205, No.3, pp. 881-885, ISSN 0014-2956

Parrott, S.; Jones, S. & Cooper, R.A. (1987). 2-Phenylethylamine catabolism by *Escherichia coli* K12. *Journal of General Microbiology*, Vol.133, No.2, pp. 347-351, ISSN 0022-1287

Reid, M. F. & Fewson, C. A. (1994). Molecular characterization of microbial alcohol dehydrogenases. *Critical Reviews in Microbiology*, Vol.20, No.1, pp. 13-56 ISSN 1040-841X

Riebel, B.R.; Gibbs, P.R.; Wellborn, W.B. & Bommarius, A.S. (2002). Cofactor regeneration of NAD^+ from NADH: novel water-forming NADH oxidase. *Advanced Synthesis and Catalysis*, Vol.344, No.10, pp. 1156-1168, ISSN 1615-4150

Riebel, B.R.; Gibbs, P.R.; Wellborn, W.B. & Bommarius, A.S. (2003). Cofactor regeneration of both NAD^+ from NADH and $NADP^+$ from NADPH:NADH oxidase from *Lactobacillus sanfranciscensis*. *Advanced Synthesis and Catalysis*, Vol.345, No.6+7, pp. 707-712, ISSN 1615-4150

Romano, A.; Gandolfi, R.; Nitti, P.; Rollini, M. & Molonari, F. (2002). Acetic acid bacteria as enantioselective biocatalysts. *Journal of Molecular Catalysis B: Enzymatic*, Vol.17, No.6, pp. 235-240, ISSN 1381-1177

Ross, R.P. & Claiborne, A. (1992). Molecular cloning and analysis of the gene encoding the NADH oxidase from *Streptococcus faecalis* 10C1. Comparison with NADH peroxidase and the flavoprotein disulfide reductases. *Journal of Molecular Biology*, Vol.227, No.3, pp. 658-671, ISSN 0022-2836

Schmidt, H.L.; Stocklein, W.; Danzer, J.; Kirch, P. & Limbach, B. (1986). Isolation and properties of an H_2O-forming NADH oxidase from *Streptococcus faecalis*. *European Journal of Biochemistry*, Vol.156, No.1, pp. 149-155, ISSN 0014-2956

Shaw, J. P. & Harayama, S. (1990). Purification and characterization of TOL plasmid-encoded benzyl alcohol dehydrogenase and benzaldehyde dehydrogenase of *Pseudomonas putida*. *European Journal of Biochemistry*, Vol.191, No.3, pp. 705-714, ISSN 0014-2956

Sheldon, R.A.; Arends, I.W.C.E. & Dijksman, A. (2000). New developments in catalytic alcohol oxidations for fine chemicals synthesis. *Catalysis Today*, Vol.57, No.1-2, pp. 157-166, ISSN 0920-5861

Tamura, Y.; Ohkubo, A.; Iwai, S.; Wada, Y.; Shinoda, T.; Arai, K.; Mineki, S.; Iida, M. & Taguchi, H. (2002). Two forms of NAD-dependent D-mandelate dehydrogenase in *Enterococcus faecalis* IAM 10071. *Applied and Environmental Microbiology*, Vol.68, No.2, pp. 947-951, ISSN 0099-2240

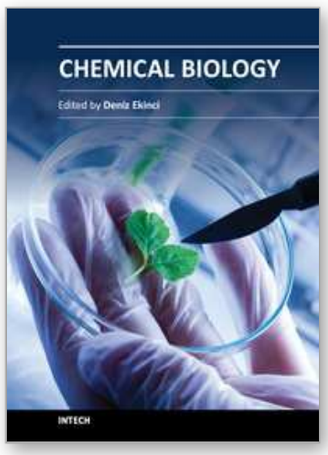
Toomey, D. &Mayhew, S.G. (1998). Purification and characterisation of NADH oxidase from *Thermus aquaticus* YT-1 and evidence that it functions in a peroxide-reduction system. *European Journal of Biochemistry*, Vol.251, No.3, pp. 935-945, ISSN 0014-2956

Tsuchiya, S.; Miyamoto, K. & Ohta, H. (1992). Highly efficient conversion of (±)-madelic acid to its (*R*)-enantiomer by combination of enzyme-mediated oxidation and reduction. *Biotechnology Letters*, Vol.14, No.12, pp. 1137-1142, ISSN 0141-5492

Wang, X.; Mann, C.J.; Bai, Y.; Ni, L. & Weiner, H. (1998). Molecular cloning, characterization, and potential roles of cytosolic and mitochondrial aldehyde dehydrogenases in ethanol metabolism in *Saccharomyces cerevisiae*. *Journal of Bacteriology*, Vol.180, No.4, pp. 822-830, ISSN 0021-9193

Yang, X. & Ma, K. (2005). Purification and characterization of an NADH oxidase from extremely thermophilic anaerobic bacterium *Thermotoga hypogea*. *Archives of Microbiology*, Vol.183, No.5, pp. 331-337, ISSN 0302-8933

Yang, X. & Ma, K. (2007). Characterization of an exceedingly active NADH oxidase from the anaerobic hyperthermophilic bacterium *Thermotoga maritima*. *Journal of Bacteriology*, Vol.189, No.8, pp. 3312-3317, ISSN 0021-9193



Chemical Biology

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Chemical biology utilizes chemical principles to modulate systems to either investigate the underlying biology or create new function. Over recent years, chemical biology has received particular attention of many scientists in the life sciences from botany to medicine. This book contains an overview focusing on the research area of protein purification, enzymology, vitamins, antioxidants, biotransformation, gene delivery, signaling, regulation and organization. Particular emphasis is devoted to both theoretical and experimental aspects. The textbook is written by international scientists with expertise in synthetic chemistry, protein biochemistry, enzymology, molecular biology, drug discovery and genetics many of which are active chemical, biochemical and biomedical research. The textbook is expected to enhance the knowledge of scientists in the complexities of chemical and biological approaches and stimulate both professionals and students to dedicate part of their future research in understanding relevant mechanisms and applications of chemical biology.

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